



# Differential role of angiotensin II receptor subtypes on endothelial superoxide formation

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**1** The physiological role of the angiotensin II AT<sub>2</sub> receptor subtype is not fully characterized. We studied whether AT<sub>2</sub> receptor could antagonize AT<sub>1</sub> mediated superoxide formation in endothelial cells.

**2** In quiescent human umbilical vein endothelial cells (HUVEC) superoxide formation was measured after long-term incubation (6 h) with angiotensin II in the presence or absence of its receptor blocker candesartan (AT<sub>1</sub>) or PD123319 (AT<sub>2</sub>) using the cytochrome c assay. In separate experiments, the effects of AT<sub>2</sub> mediated effects on activities of cellular phosphatases including the src homology 2 domain containing phosphatases (SHP-1) was studied.

**3** The basal superoxide formation ( $0.19 \pm 0.03$  nmol superoxide mg protein<sup>-1</sup> min<sup>-1</sup>) in HUVEC was increased by 37.1% after exposure to angiotensin II (100 nM) which was due to an activation of a NAD(P)H oxidase. This was abolished by candesartan (1  $\mu$ M) as well as the tyrosine kinase inhibitor genistein. In contrast, blockade of AT<sub>2</sub> receptors by PD123319 enhanced the superoxide formation by 73.7% in intact cells. Stimulation of AT<sub>2</sub> went along with an increased activity of tyrosine phosphatases in total cell lysates (29.8%) and, in particular, a marked stimulation of src homology 2 domain containing phosphatases (SHP-1, by 293.4%). The tyrosine phosphatase inhibitor vanadate, in turn, prevented the AT<sub>2</sub> mediated effects on superoxide formation. The expression of both angiotensin II receptor subtypes AT<sub>1</sub> and AT<sub>2</sub> was confirmed by RT–PCR analysis.

**4** It is concluded that AT<sub>2</sub> functionally antagonizes the AT<sub>1</sub> induced endothelial superoxide formation by a pathway involving tyrosine phosphatases.

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**Keywords:** Angiotensin II receptor subtypes; superoxide anion; endothelial cell physiology; tyrosine phosphatases; Src homology 2 domain containing phosphatases

**Abbreviations:** AngII, Angiotensin II; CV, CV11974; DPI, diphenyleniodonium chloride; HUVEC, human umbilical vein endothelial cells; L-NA, N<sup>G</sup>-nitro-L-arginine; PAO, phenylarsine oxide; PD, PD123319; PTP, phosphotyrosine phosphatases; SHP-1, src homology 2 domain containing phosphatases; SOD, superoxide dismutase

## Introduction

Angiotensin II (AngII) plays an important role in the pathogenesis of cardiovascular disorders such as development of myocardial and smooth muscle hypertrophy, arteriosclerosis and neointima formation after balloon injury (Helin *et al.*, 1997). This is, at least in part, due to an AngII-induced formation of superoxide anions in vascular tissues (Griendling *et al.*, 1994; Pagano *et al.*, 1998). Superoxide scavenges NO and therefore abolishes its vasoprotective effects resulting in an impairment of endothelium dependent dilation, enhanced platelet aggregation and intimal proliferation (Mügge *et al.*, 1991; Ohara *et al.*, 1993). The inactivation of NO by superoxide may be particularly important in endothelial cells (Ohara *et al.*, 1993; Warnholtz *et al.*, 1999) where this autacoid is synthesized.

Virtually all stimulating effects of AngII on cardiovascular cells are mediated by the AT<sub>1</sub> receptor subtype. The physiological effects of AT<sub>2</sub> receptor stimulation are, however, less clear (Horiuchi *et al.*, 1999). Experimental data suggest that the AT<sub>2</sub> receptor opposes some AT<sub>1</sub> receptor mediated effects on cell growth as well as on water and electrolyte balance. (Munzenmaier & Greene, 1996; Hein *et al.*, 1995). The AT<sub>1</sub> in vascular cells is coupled *via* G-proteins to the inositol

1,4,5-triphosphate pathway, and additionally, to cytoplasmatic tyrosine kinases such as the JAK/STAT pathway (Schieffer *et al.*, 1996), while the signal pathway of the AT<sub>2</sub> mediated effects is not fully understood (Horiuchi *et al.*, 1999). It was proposed that an activation of cellular phosphotyrosine phosphatase (PTP) activity plays a key role in AT<sub>2</sub> signaling (Tsuzuki *et al.*, 1996; Bedecs *et al.*, 1997; Marrero *et al.*, 1998). Recently, the vanadate sensitive src homology 2 domain containing PTP (SHP-1, previously termed PTPIC, SHPTP-1 and SHP) which is known to inhibit growth related signalling predominantly in hemopoietic cells (Klingmuller *et al.*, 1995), has been shown to be activated by AT<sub>2</sub> stimulation in N1E-115 cells (Bedecs *et al.*, 1997). In vascular smooth muscle cells SHP-1 was responsible for JAK2 dephosphorylation and thus termination of AngII induced JAK/STAT pathway (Marrero *et al.*, 1998).

With respect to endothelial superoxide formation, it has been demonstrated recently that AngII serves as an effective stimulus, acting *via* an AT<sub>1</sub> dependent mechanism (Warnholtz *et al.*, 1999). Nothing is known, however, about a potential antagonistic role of the AT<sub>2</sub> receptor in this process. Here, we studied whether AT<sub>2</sub> stimulation counteracts the AT<sub>1</sub> mediated superoxide production in cultured endothelial cells and whether activation of PTP is involved in these AT<sub>2</sub>-dependent signaling pathways. An AT<sub>2</sub> mediated reduction of

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endothelial superoxide might play an important role in the beneficial therapeutic effect of AT1 blockers.

## Methods

### Cell culture

Endothelial cells (HUVEC) were isolated from freshly obtained human umbilical veins and grown to confluence in Medium 199 supplemented with 16% foetal calf serum and 20% endothelial growth medium. After serum starvation for 24 h the quiescent HUVEC were treated with AngII (0.1–1  $\mu\text{M}$ ) in the presence or absence of the AT1 blocker CV11974 [CV, 1  $\mu\text{M}$ , (candesartan)] or the AT2 blocker PD123319 (PD, 1  $\mu\text{M}$ ) for 6 h.

### Detection of endothelial superoxide production

Endothelial superoxide formation was determined by the cytochrome c method. HUVEC were incubated in a HEPES (20 mM)-Tyrode buffer containing cytochrome c (40  $\mu\text{M}$ ) with or without SOD (200 u  $\text{ml}^{-1}$ ). After 30 min the supernatant was removed and the SOD-inhibitable reduction of cytochrome c measured at 550 nm. The superoxide production was calculated from the difference between samples with or without SOD ( $\epsilon_{550} \text{ nM} = 21.1 \text{ mM}^{-1} \text{ cm}^{-1}$ ). NADH induced superoxide production in cell lysates was measured by adding NADH (100  $\mu\text{M}$ ) to aliquots of cell lysates.

### Immunoprecipitation and Western blot analysis

HUVEC were lysed in modified ice cold RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.1% (w  $\text{v}^{-1}$ ) SDS, 0.5% DOC, 1% NP40, 10  $\mu\text{g ml}^{-1}$  leupeptin, 10  $\mu\text{g ml}^{-1}$  pepstatin, 10  $\mu\text{M}$  PMSF, 2 mM orthovanadate, 100 mM NaF). 10  $\mu\text{g ml}^{-1}$  anti-SHP antibody was added to the lysates for 1 h at 4°C. Immunocomplexes of SHP-1 were precipitated by addition of protein G-agarose beads and allowed to equilibrate for 12 h. The precipitates were divided in two halves and either used for tyrosine phosphatase assay or for Western blotting. For the latter, the immunoprecipitated SHP-1 were separated on a SDS-PAGE following standard procedures and transferred onto nitrocellulose membrane. After incubation with the SHP-1 antibody and an alkaline phosphatase conjugated secondary antibody the loading of SHP-1 was detected using the NBT/BCIP system.

### Tyrosine phosphatase assay

For measurements of cellular tyrosine phosphatase activity a commercially available kit (Promega) was used. Endothelial cells were lysed in 100  $\mu\text{l}$  lysis buffer (20 mM Tris HCl pH 7.2, 2 mM EDTA, 1% Triton-X100, 1 mM PMSF, 0.1% 2-mercaptoethanol, 1  $\mu\text{g ml}^{-1}$  aprotinin, 1  $\mu\text{g ml}^{-1}$  leupeptin). The phosphatase reaction mix, containing 5  $\mu\text{l}$  of the tyrosine phosphopeptide DADE(pY)LIPQQG, 40  $\mu\text{l}$  storage buffer and 5  $\mu\text{l}$  of the sample were incubated for 45 min at 37°C.  $\text{PO}_4^{3-}$  concentration was determined at 595 nm using a ELISA reader (TECAN) and normalized to protein content of the sample.

### Reverse transcriptase PCR

For RT-PCR 1  $\mu\text{g}$  total RNA was reverse transcribed in (mM): Tris/HCl (pH 8.3) 50, KCl 75,  $\text{MgCl}_2$  3, DTT 10, dNTP

0.5, specific reverse primer 3 and 100 uM-MLV RT (Promega). 35 cycles of PCR were performed in (mM): Tris/HCl (pH 8.8) 50, KCl 50,  $\text{MgCl}_2$  1.5, DTT 2, 0.1% TritonX-100, dNTP 0.2, 0.7  $\mu\text{M}$  specific primers and 2.5 u Taq Pol (AmpliTaqGold, Promega). The used primers were (AT1) 5'-GCTATGGAA-TACCGCTGGCCCTTTGG-3' (265–290) and 5'-GGCATG-GCCGTGTCCACAATATCTGC-3' (854–829); (AT2): 5'-CCAGCGGTCTTCACTTCGGG-3' (44–63) and 5'-GCCA-GAACAACAGCAGCTGC-3' (791–772) from human AngII receptor mRNA.

### Drugs

CV11974 (Candesartan) was kindly provided by Astra Chemicals (Mölndal, Sweden). PD123319 was obtained as a gift from Parke Davis (Ann Arbor, U.S.A.). SOD was purchased from Boehringer MA (Germany). Endothelial cell growth medium was from PromoCell (Heidelberg, Germany) and orthovanadate from Alexis (San Diego, U.S.A.). Anti-phosphotyrosine was purchased from Upstate (Lake Placid, U.S.A.), anti-SHP-1 from Santa Cruz (Santa Cruz, U.S.A.) and the tyrosine phosphatase activity assay kit from Promega (Madison, U.S.A.). All other drugs were obtained from Sigma (Deisenhofen, Germany).

### Statistical analysis

Statistical comparisons with and without treatment within the same experimental group were performed using the Wilcoxon signed rank test for paired observations. Differences were considered significant at an error probability of  $P < 0.05$ . For descriptive means all results are expressed as means  $\pm$  s.e.mean.

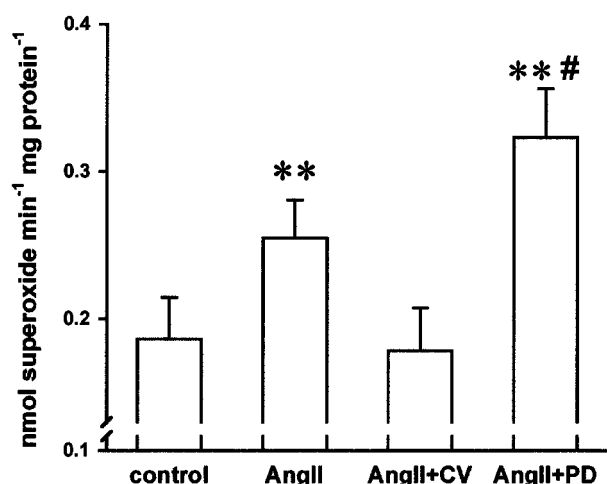
## Results

### Basal superoxide production in endothelial cells

Quiescent cells produced  $0.27 \pm 0.03$  nmol superoxide  $\text{mg protein}^{-1} \text{ min}^{-1}$  ( $n = 10$ ). Since a pretreatment with the NO-synthase inhibitor  $\text{N}^G$ -nitro-L-arginine (L-NA, 30  $\mu\text{M}$ ) increased the basal superoxide production by a further 48.5% ( $n = 10$ ,  $P < 0.01$ ), all further experiments were performed in the presence of L-NA to avoid modulating effects of NO. Proliferating cells kept in serum had an about 3 fold higher basal superoxide formation ( $0.27 \pm 0.03$  vs  $0.84 \pm 0.07$  nmol superoxide  $\text{mg protein}^{-1} \text{ min}^{-1}$ ,  $n = 10$ ,  $P < 0.01$ ).

### Different effects of AT1 and AT2 stimulation on endothelial superoxide formation

AngII (100 nM) did not affect the superoxide formation in proliferating cells ( $0.75 \pm 0.05$  vs  $0.85 \pm 0.04$  nmol superoxide  $\text{mg protein}^{-1} \text{ min}^{-1}$ ,  $n = 11$ , n.s.). In quiescent cells, however, exposure to AngII (10–100 nM) for 6 h significantly enhanced the superoxide formation by 30.0 and 37.1% ( $n = 12$ ,  $P < 0.05$ ), respectively, whereas at a higher concentration (1  $\mu\text{M}$ ) of AngII did not significantly affect the superoxide formation. The AngII (0.1  $\mu\text{M}$ ) mediated superoxide formation was mediated by AT1 receptor, since the AT1 blocker CV (1  $\mu\text{M}$ ) abolished the AngII induced effect (Figure 1,  $n = 24$ ,  $P < 0.01$ ). In contrast, blockade of the AT2 by PD (1  $\mu\text{M}$ ) augmented the superoxide formation by 73.7% of control (Figure 1,  $n = 24$ ,  $P < 0.01$ ). Although this effect was highly significant in some subset of cells, PD123319 showed no significant effects on AngII induced superoxide formation. In the latter cells,



**Figure 1** Angiotensin II (AngII, 100 nM incubation for 6 h increased the superoxide formation in intact endothelial cells. This was inhibited by the AT<sub>1</sub> receptor blocker CV11974 (CV, 1  $\mu$ M) whereas an AT<sub>2</sub> receptor (PD123319, 1  $\mu$ M) blockade further increased the superoxide formation. ( $n=24$ , \*\* $P<0.01$  vs. control, # $P<0.05$  vs angiotensin II).

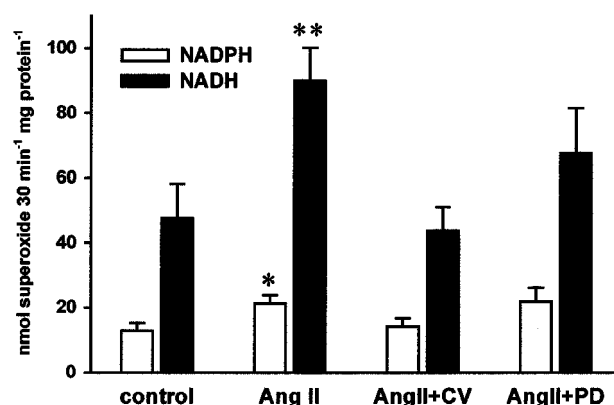
however, AngII induced a markedly stronger increase in superoxide production (29.7% vs 95.0%) supporting the inhibitory role of AT<sub>2</sub> receptors on superoxide formation. The flavoprotein enzyme inhibitor diphenyliodonium chloride (DPI, 10  $\mu$ M) as well as the direct NAD(P)H oxidase blocker phenylarsine oxide (PAO, 1  $\mu$ M) (Le Cabec & Maridonneau, 1995) significantly attenuated the AngII induced superoxide formation by 79.3 and 67.3%, respectively ( $n=6$ ,  $P<0.05$ ). As shown in Figure 2, pre-stimulation of the AT<sub>1</sub> receptor in intact cells also augmented the NADH and NADPH-dependent superoxide formation after cell lysis by 88.7% ( $n=10$ ,  $P<0.01$ ) and 65.1% ( $n=17$ ,  $P<0.05$ ), respectively. This was, in turn, prevented by preincubation of the intact cells with the AT<sub>1</sub> blocker CV whereas an AT<sub>2</sub> blockade failed to have any effects.

#### *Tyrosine kinases mediate AT<sub>1</sub> induced endothelial superoxide production*

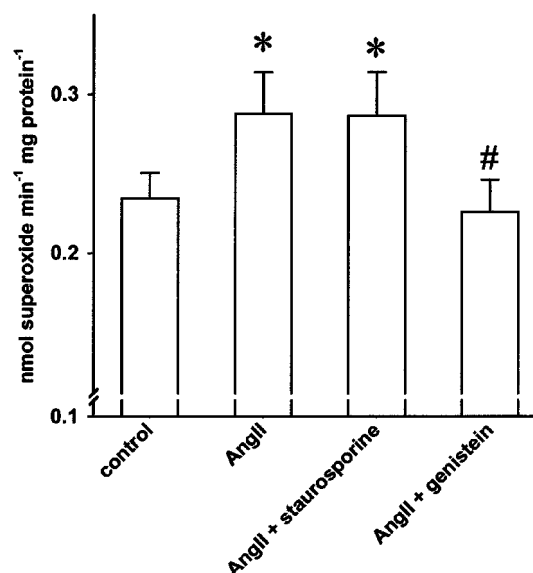
As shown in Figure 3 the AngII induced superoxide formation could be prevented by the tyrosine kinase inhibitor genistein (30  $\mu$ M,  $n=8$ ,  $P<0.05$ ) while the protein kinase C inhibitor staurosporine (100 nM) was not effective. The latter concentration of staurosporine inhibited phorbol 12-myristate 13-acetate induced superoxide formation in cells cultured in same conditions (Sohn *et al.*, 1999). In separate experiments, the more specific PKC-blocker chelerythrine chloride (10  $\mu$ M) was tested. It also did not exert a significant effect on AngII induced superoxide formation (control:  $0.23 \pm 0.04$  vs AngII:  $0.36 \pm 0.04$  and AngII + chelerythrine chloride:  $0.35 \pm 0.05$  nmol superoxide mg protein<sup>-1</sup> min<sup>-1</sup>,  $n=12$ , n.s.). Neither genistein nor staurosporine alone affected the basal superoxide formation ( $0.28 \pm 0.03$  vs  $0.26 \pm 0.02$  and  $0.31 \pm 0.02$  nmol superoxide mg protein<sup>-1</sup> min<sup>-1</sup>,  $n=13$ , n.s.).

#### *AT<sub>2</sub> mediates activation of PTPs*

In quiescent cells the PTP activity was enhanced by 49.8% compared to proliferating cells ( $n=18$ ,  $P<0.05$ , data not shown). Selective stimulation of AT<sub>2</sub> receptors for 15 minutes (AngII: 0.1  $\mu$ M) in the presence of the AT<sub>1</sub> receptor blocker CV (1  $\mu$ M) significantly increased the PTP-activity in quiescent



**Figure 2** In separate experiments the pretreated endothelial cells were resuspended in a lysis buffer and either the NADPH or NADH (100  $\mu$ M each) dependent superoxide formation measured ( $n=17$  for NADPH and  $n=10$  for NADH; \* $P<0.05$  and \*\* $P<0.01$  vs control). In contrast to intact cells an AT<sub>2</sub> receptor inhibition did not affect the AT<sub>1</sub> receptor mediated stimulation of NADH/NADPH dependent superoxide production.

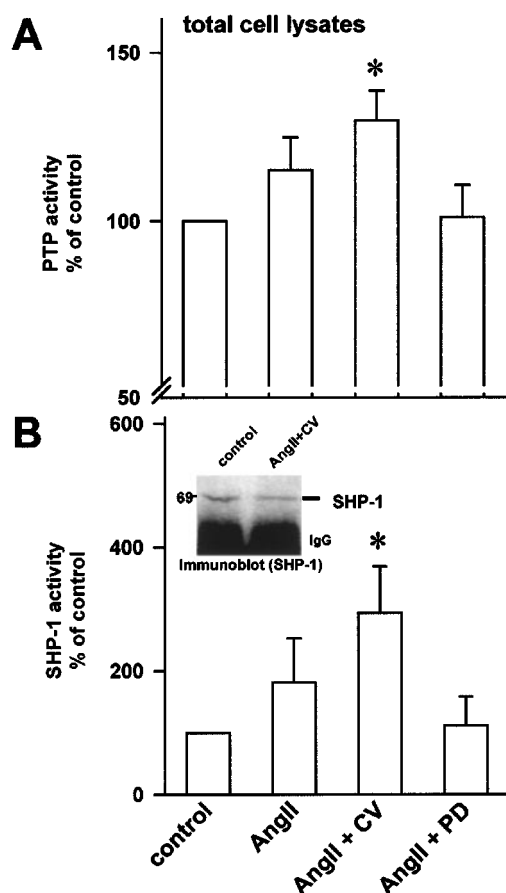


**Figure 3** The angiotensin II (AngII, 100 nM) induced endothelial superoxide formation was abolished in the presence of the tyrosine kinase inhibitor genistein (30  $\mu$ M,  $n=11$ , \* $P<0.05$  vs control, # $P<0.05$  vs angiotensin II) while the protein kinase C inhibitors staurosporine and chelerythrine chloride (10  $\mu$ M, see text) were not effective.

cells by a further 29.8% (Figure 4A,  $P<0.05$ ,  $n=10$ ). To evaluate the potential role of a particular PTP in AT<sub>2</sub> receptor mediated inhibition of superoxide formation, we assessed the activity of SHP-1, which is involved in AT<sub>2</sub> receptor mediated JAK2 dephosphorylation (Marrero *et al.*, 1998). Stimulation of the AT<sub>2</sub> receptor markedly increased the SHP-1 activity by 293.4% (Figure 4B,  $n=6$ ,  $P<0.05$ ). Equal loading of SHP-1 of the PTP-assay was confirmed by Western blotting of immunoprecipitated SHP-1 (see Figure 4B inlet).

#### *PTP inhibition prevents AT<sub>2</sub> mediated effects on endothelial superoxide production*

Using the PTP inhibitor vanadate we studied whether changes in PTP activity modulate endothelial superoxide production.



**Figure 4** (A) Activity of cellular phosphotyrosine phosphatases (PTP). Selective stimulation of endothelial AT<sub>2</sub> receptors (in the presence of the AT<sub>1</sub> receptor blocker CV, 1  $\mu$ M) increased the activity of phosphotyrosine phosphatases measured in total endothelial cell lysates ( $n=10$ ,  $*P<0.05$  vs control). (B) In separate experiments, SHP-1 was immunoprecipitated from cell lysates and its activity determined. AT<sub>2</sub> receptor stimulation markedly enhanced the SHP-1 activity ( $n=6$ ,  $*P<0.05$  vs control). Inset, Immunoblot of SHP-1. Aliquots from immunoprecipitated SHP-1 were subjected to SDS-PAGE and blotted onto nitrocellulose membrane. Equal loading of SHP-1 was confirmed by specific antibody against SHP-1.

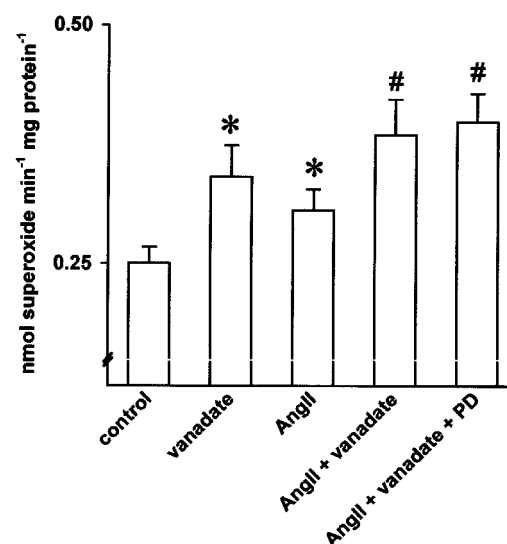
We showed previously (Sohn *et al.*, 1999) that vanadate at a concentration of 100  $\mu$ M significantly increases tyrosine phosphorylation of various proteins in endothelial cells. The same concentration of vanadate also enhanced the basal endothelial superoxide generation (by 35.9%,  $P<0.05$ , Figure 5). Vanadate further augmented the AT<sub>1</sub> induced superoxide formation ( $P<0.05$ ), while an AT<sub>2</sub> receptor blockade did not further affect the superoxide production in the presence of vanadate ( $n=15$ , Figure 5).

#### AngII receptor subtypes in HUVEC

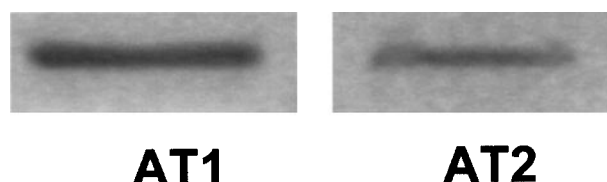
The expression of both AngII receptor subtypes was confirmed by RT-PCR analysis (Figure 6). In contrast to AT<sub>1</sub>, the AT<sub>2</sub> receptor was found to be not expressed in some populations of HUVEC.

## Discussion

This study shows that AngII *via* AT<sub>1</sub> receptor induces superoxide formation in endothelial cells (Zhang *et al.*, 1999). This is in agreement with previous studies in vascular



**Figure 5** Effect of the phosphotyrosine phosphatase inhibitor vanadate on angiotensin II induced endothelial superoxide formation. Vanadate (100  $\mu$ M) further augmented the angiotensin II mediated superoxide formation. AT<sub>2</sub> receptor blockade (PD123319, 1  $\mu$ M), however, did not affect the superoxide production in the presence of vanadate ( $n=15$ ,  $*P<0.05$  vs control,  $\#P<0.05$  vs angiotensin II).



**Figure 6** Expression of AT<sub>1</sub> and AT<sub>2</sub> receptors in HUVEC. RT-PCR analysis showed the expression of both angiotensin II receptor subtypes in HUVEC.

smooth muscle cells (Griendling *et al.*, 1994) and fibroblasts (Pagano *et al.*, 1998). The inhibitory effects of DPI and PAO (Le Cabec & Maridonneau, 1995), as well as the NADH-sensitivity of superoxide formation suggest that a NAD(P)H dependent oxidase was the main source of endothelial superoxide formation in our experiments (Jones *et al.*, 1996; Al-Mehdi *et al.*, 1998).

The regulation of this superoxide generating system in endothelial cells is not fully understood. Increases in intracellular calcium and/or activation of protein kinase C seem to play a role (Matsubara & Ziff, 1986). Our results indicate, however, that the AT<sub>1</sub> mediated superoxide formation in HUVEC includes a tyrosine phosphorylation dependent step (Sohn *et al.*, 2000), since genistein, but not staurosporine, completely abolished the effect of AngII. This is in agreement with studies in neutrophils (Dusi *et al.*, 1996) and fibroblasts (Thannickal *et al.*, 1998) showing tyrosine phosphorylation being an important pathway in the activation of free radical production.

On the other hand, a blockade of the AT<sub>2</sub> receptor resulted in a further increase of the AT<sub>1</sub> mediated superoxide formation, suggesting an antagonistic function of both receptor subtypes. Such an antagonism have been reported previously on bFGF induced proliferation of cultured rat coronary endothelial cells (Stoll *et al.*, 1995). Likewise, long-term treatment with AngII resulted in an increase of the capillary density in the rat cremaster muscle, which was prevented by blockade of AT<sub>1</sub>, but was further augmented by an AT<sub>2</sub> inhibition (Munzenmaier & Greene, 1996). Since AT<sub>2</sub>

activation increases PTP activity in various cells (Tsuzuki *et al.*, 1996; Bedecs *et al.*, 1997; Horiuchi *et al.*, 1999) we studied whether PTPs play a role in regulation of endothelial superoxide production. In this study, the PTP-inhibitor vanadate significantly increased the superoxide generation suggesting that, in turn, an increased activity of PTP results in an attenuation of superoxide formation. Direct measurements of PTP-activity confirmed that AT2 stimulation indeed activates PTP. Accordingly, in quiescent cells with a low rate of superoxide formation, the PTP activity was *per se* elevated. Tsuzuki *et al.* (1996) observed in R3T3 cells an AT2 mediated inhibition of cell proliferation which was associated with an activation of PTP. Possible candidates might represent, in particular, the vanadate sensitive SHP-1 which is involved in termination of proliferation related signalling by growth factors or by AngII (Bedecs *et al.*, 1997; Marrero *et al.*, 1998; Klingmuller *et al.*, 1995). We demonstrated that the AT2 induced inhibition of superoxide production is associated with a marked increase of SHP-1 activity. Although these studies do not establish a causal relationship, the findings are highly suggestive for a role of SHP-1 in regulation of endothelial superoxide formation. This is further supported by recent findings in neutrophils showing that an initiation of the respiratory burst was followed by a declined activity of SHP-1 (Brumell *et al.*, 1997).

Incubation of intact HUVEC with AngII also increased the NADH dependent superoxide formation in cell homogenates, which was mediated by AT1 receptors. AT2 receptors do not seem to affect the NAD(P)H oxidase assembly but rather control its activity *via* modulating PTP which seem not to function anymore in cell lysates. Accordingly, inhibition of PTP by vanadate abolished the effect of an AT2 blockade on

superoxide generation in intact cells but this effect did not persist after lysis of the cells.

Some investigators were unable to detect an effect on AngII induced superoxide formation by AT2 receptor blockade. The expression of the AT2 receptor is quite variable in different tissues and appears not only to be dependent on the cell types but also on *in vitro* culture conditions (Ichiki *et al.*, 1995; Dimmeler *et al.*, 1997; Pueyo & Michel, 1997). Although the HUVEC were kept under similar conditions in our study the expression of AT2 was not found in some populations. However, it supports our hypothesis of an inhibitory role of AT2 receptors on superoxide formation, that in HUVEC lacking the AT2 receptor, the AngII induced superoxide formation was markedly stronger.

In conclusion, the present experiments demonstrate that AngII receptor subtypes differentially modulate endothelial superoxide formation. While AT1 activates superoxide formation, AT2 receptors appear to attenuate this AT1-induced effect, most likely by its functional coupling to activation of PTP, probably SHP-1. The relative distribution of both receptor subtypes on the same cell type, which may vary following ischemia or in myocardial hypertrophy (Bartunek *et al.*, 1999; Horiuchi *et al.*, 1999) may therefore influence endothelial superoxide formation and hence NO dependent dilation.

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